

UNIVERSIDAD SAN FRANCISCO DE QUITO
Colegio de Postgrados

**“Evidence for a Modified Th2-like response during chronic
helminth infections in school-age children from the rural
tropics of Ecuador”**

Respuesta inmune del tipo Th2 modificada durante la infección
crónica por helmintos en niños de edad escolar que viven en
comunidades rurales y tropicales del Ecuador

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Tesis de grado presentada como requisito para la obtención
del título de Magíster en Microbiología

Quito, Mayo del 2009

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HOJA DE APROBACIÓN DE TESIS

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Quito, Mayo del 2009

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*To God
To Humanity
To my beloved Mother
To You*

Dear Father:

I would like to thank You for all the things I have received from Thee. Life is a strange little word that encloses too much meaning that one could not be able to describe even if all words were to be used. Nonetheless is this life what I appreciate the most, is this life the main gift I have been granted and I hope not to misuse such a great opportunity but to pay back with all my effort wondering if it will, someday, be translated into more justice, into less suffering.

I know I might not be the best of Your sons and I sincerely apologize for not trying hard enough. However, I would like You to receive this meaningless word as a demonstration of my love for You, and thus for all human race.

I hope You can give me a hand and strength all the attitudes and abilities I need to be able to accomplish the objectives planned.

With all my love,

Miguel

PS:

אני אוהב אותך

Acknowledgements

It would be impossible to list all people who, in many different ways, made of this work a reality today without forgetting to mention some important contributions. However, I will take the risk and beforehand apologize for any involuntary missing.

Special thanks must be given to Philip Cooper for his commitment with this project from the very beginning a support that lasted until today; to Prof. Gordon Dougan for hosting me so warmly in the UK; to Prof. David Lewis for his always opportune advice; to Prof. George Griffin for his sincerity and hospitality; to Fernanda Schreiber for her key collaboration during the final steps of the project; to Nely Broncano and Susana Benítez for their valuable help in field work execution; to Bernadette Mah for her wonderful work coordinating a global-wide community which included me; to Bela Woodrow for being an important help while visiting St. George's; to all people who work at Laboratorio de Investigaciones Clínicas - Quinindé for their support; to all people from Quinindé and Las Golondrinas who indirectly gave a great hand, including, Dr . Augusto Rodríguez, Sra. Sandra, Sra. Marcia, Lic. Molina, Lic. Bravo among others; to all my class-mates in USFQ for being the right word in the right moment, specially to Paul, Gabriela, Patricio, Rommy and Pablo; to Gabriel for believing that I was a good candidate for this course, I hope I did not disappointed you; to Manuel for being such a great guide during the whole process; to my mother and brothers for being that fresh burst of air always needed; to you my beloved lady for standing next to me even when I inadvertently ignore how important your love is; to God, the only one.

Resumen

La infección por geohelminthos es un problema de salud pública, especialmente en regiones con climas tropicales y subtropicales. Se ha observado una relación epidemiológica entre helmintiasis y frecuencia de fenómenos alérgicos, aunque estudios con resultados opuestos también han sido publicados. La respuesta T_H2 modificada es un fenotipo inmunológico caracterizado por altos niveles de IL-10 con incremento concomitante en los niveles de IgG4 que ocurren sobre la base de una respuesta T_H2 (altos niveles de IL-4, IL-5, IL-13 y de anticuerpos tipo IgE). Estudios experimentales en ratones han demostrado que la estimulación inmune con helmintos puede inducir la implementación de una respuesta T_H2 modificada. Esta respuesta ha sido demostrada en humanos únicamente en el contexto de exposición a altas concentraciones de alérgeno del gato. En el presente estudio piloto (n = 60) se investigó si la exposición a infecciones por geohelminthos con alta carga parasitaria y con marcadores serológicos que sugieren cronicidad se encuentra asociada a la presencia de una respuesta de tipo T_H2 modificada, y si esta difiere del tipo de respuesta inmune asociada a las infecciones de baja carga parasitaria. Se encontró que las infecciones con alta carga parasitaria se asocian a la expresión de un patrón de citocinas similar (altos niveles de IL-10) al de la respuesta T_H2 previamente descrita mientras que en las infecciones con baja carga parasitaria se observó una respuesta de citocinas con un patrón de respuesta del tipo T_H2 clásico (altos niveles de IL-5 e IL-13 con bajos niveles de IL-10). Estos datos sugieren que durante la infección crónica con helmintos se podrían estar activando ciertos mecanismos tipo T_H2 modificada que eventualmente podrían tener un efecto inmunomodulador. Esto, a su vez, podría explicar la divergente asociación entre infecciones por helmintos y fenómenos alérgicos (las infecciones agudas exacerbarían, mientras que las crónicas disminuirían la frecuencia de fenómenos alérgicos)

Abstract

Geohelminths are a public health issue, specially in low-income tropical and subtropical regions. An inverse epidemiologic relationship between geohelminth infection and the frequency of allergic conditions has been reported, although some reports showing the opposite have also been published. The modified T_H2 response is an immune phenotype characterized by increased IL-10 levels with concomitant high IgG4 in the context of a T_H2-type response; i.e, IL-4, IL-5, IL-13 and IgE antibodies. Experimental studies have shown the induction of a modified T_H2 response during murine helminth infections. This response has been observed in humans only in during high-dose exposure to cat allergen. In this small pilot study (n = 60) it was investigated whether exposure to high burden helminth infection in which serological markers of chronicity were present was associated to the presence of a modified T_H2 response, and whether this response was different to the one observed during low burden helminth infections. It was observed that high burden helminth infections were associated to a modified T_H2-like cytokine response (high IL-10 levels) whereas low burden infections were associated to a classic T_H2-type cytokine response (high IL-5, IL-13 with concomitant low IL-10 levels). These data suggest that during chronic/high burden helminth infections modified T_H2 mechanisms might be setting up which eventually could lead to immune response modulation. These could explain ambiguous associations between helminth infections and allergen conditions (i.e., acute infections would increase whereas chronic infection would decrease the frequency of allergic phenomena).

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Abbreviations

BFS	Bovine Foetal Serum
CD	Cluster of Differentiation
CEP	Cytokine Expression Profile
DC	Dendritic Cells
DNA	Deoxyribonucleic acid
ES	Excretory/Secretory (products)
FA	Factor Analysis
FEC	Faecal Egg Count
IFN- β	Interferon β
IFN- γ	Interferon γ
GALT	Gut Associated Lymphocyte Tissue
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G, subtype 1
IgG4	Immunoglobulin G, subtype 4
IL	Interleukin
mT _H 2	Modified T _H 2 response
PAMPs	Pathogen Associated Molecular Patterns
PCA	Principal Component Analysis
PBMC's	Peripheral Blood Mononuclear Cells
PPD	Purified Protein Derivative (Tuberculin)
RNA	Ribonucleic Acid
SEB	<i>Staphylococcus aureus</i> Enterotoxin B
SS 0.9%	Saline Solution at 0.9%
TGF- β	Transforming Growth Factor β
T _H 1	Type 1 Helper (T Cell)
T _H 2	Type 2 Helper (T Cell)
TNF- α	Tumour Necrosis Factor α
T _{REG}	Regulatory T Cells
WHO	World Health Organization
WTSI	Wellcome Trust Sanger Institute

Introduction

1.1 Epidemiology of geohelminth infections.

Geohelminths, or soil-transmitted helminths, are estimated to infect more than two billion people worldwide, especially in low-income tropical areas where infections might be highly prevalent [1, 2, 3, 4]. *Ascaris lumbricoides*

infects nearly one billion people around the globe [5, 6] and causes more than 60000 deaths per year, mostly in children [2, 7]. Roundworms are an important cause of morbidity and it is calculated that in 1990 DALYs (Disability Adjusted Life Years) due to ascariasis were 10.5 million [8]. Altogether, geohelminths account for 39 million DALYs [8] as compared to malaria (35.7 million) [9], tuberculosis (28.4 million), and HIV (68.7 million) [10].

1.2 Epidemiological association between geohelminth infections and allergic disorders: the revised hygiene hypothesis.

The observation that endemically helminth-infected populations have low incidence of allergic diseases [11, 12] has led to the hypothesis that low immune stimulation (including from helminthes) may result in increased prevalence of allergic conditions in developed nations, an important change in the original statement of the hygiene hypothesis, which indicated that deficient Th1 stimulation in urbanized populations led to increased prevalence of allergic conditions [12, 13, 14].

In contrast, other studies have found that *Ascaris* infections enhance allergic manifestations [11, 15] suggesting a more complex setting in which exposure lengths and intensity of infection may play a role. Acute or sporadic infections might be followed by an increase in allergic symptoms while chronic or persistent infections could be associated with impaired allergic manifestations [11].

1.3 Immune response to geohelminths

Geohelminth infections induce protective T_H2 -type immune responses and they are mediated by IL-4, IL-5, IL-9, and IL-13 [12, 17, 18]. Mechanisms conferring protection are IL-5-induced eosinophilia, IgA, IgE, mastocytosis, IL-13-induced increased mucus secretion, enhanced mast cell and basophil development, IL-4 and IL-13-induced increased mucosal permeability and increased smooth muscle contractility [17, 19].

Helminth parasites may cause long-term infections in humans due to their long life span (e.g., *Ascaris lumbricoides* may live up to 2 years) and to the repeated infections occurring in endemic areas [20, 21, 22]. Long-term parasitism is likely to be achieved through different mechanisms: 1) mast cell saturation, where polyclonal IgE could saturate mast cell surface receptors limiting parasite-specific IgE binding and thus degranulation [32]; 2) induction of blocking IgG4 antibodies, where production of IgG4 antibodies targeting the same parasite epitopes as IgE could be induced resulting in limited activation of effector mechanisms [23]; 3) innate immunity variation; 4) induction of regulatory T cells (and modulatory cytokines); 5) parasite-derived immunomodulatory molecules; 6) induction of alternatively activated macrophages; and, 7) modified T_H2 response [14, 17, 19, 20, 23, 25]. At least some of these mechanisms may also affect immune responses to non-parasite antigens through by-stander effects [17] which could explain the epidemiological relationship between helminth infections and allergic disorders.

1.4 The modified T_H2 (m T_H2) response

The modified T_H2 response is an immunological phenotype that was originally described in children with high level indoor exposure to the cat allergen Fel d 1 [26]. In that study, 226 children aged 12-14 years old with or without asthma manifestations and evidence of bronchial hyper-reactivity were selected for cat-allergen specific antibodies (IgG, IgG4) measurement and these results were compared with sensitization (defined either by skin prick test or by RAST-measured IgE specific antibodies) and allergen concentration in house dust samples [26]. Authors found a phenotype characterized by increased levels of IgG, including IgG4, to Fel d 1 and a lack of cat allergen sensitization (skin prick test responses < 4 mm or low levels of Fel d 1-specific, RAST-measured IgE antibodies) in children with high level exposures to Fel d 1, the cat allergen (Figure 1) [26].

The term “modified” T_H2 response was coined due to:

- 1) The lack of delayed type hypersensitivity responses to cat allergen or other common inhalant allergens which rules out deviation towards T_H1 type immunity [26];
- 2) The presence of high IgG4 and low IgE antibodies;
- 3) The fact that this antibody profile may be found during T_H2 responses, specially when IL-10 and IL-4 are both present [27, 29]; and,
- 4) Since it has been shown that Fel d 1 induces peripheral blood mononuclear cells to produce higher IL-10 and IL-5 with lower IL-13 and IFN- γ than responses elicited by other stimulus (Tetanus toxoid) [30].

Modified T_H2 responses may be mechanisms of tolerance during high level exposure to cat allergen but not to other inhalant allergens where greater levels of exposure are associated to greater sensitisation [26]. This difference could be due to different chemical nature of allergens, different exposure time to the allergens and may be also influenced by genetic traits (MHC haplotype) [28, 30].

Finally, it must be emphasized that the modified T_H2 response could be just one of several possible “tolerogenic” mechanisms acting in individuals exposed but not sensitized to allergens (Fel d 1) since there are “non-modified” responders with no evidence of allergic diseases [28, 30].

1.5 The modified T_H2 response and helminth infections

There is experimental evidence showing the induction of a cytokine profile consistent with modified Th2 response occurring in the lungs of *Schistosoma mansoni* infected mice [31]. In this study, mice were infected with *Schistosoma mansoni* and were subsequently sensitized with OVA before airway challenging with the same antigen [31]. When comparing the difference in the levels of cytokines from bronchoalveolar lavage it was found that OVA-sensitized worm-infected mice had increased IL-4, IL-13 and IL-10 and lower IL-5 than OVA-sensitized uninfected mice [31].

Justification

Chronic Diseases, including allergies, are increasingly been recognized as important causes of morbidity and death worldwide. It is estimated that 60% (35 million) of deaths in 2005 were attributable to these conditions (Bousquet et al,

2007). Chronic Respiratory Diseases (CRD), which include asthma and other forms of respiratory allergies, are important public health issues worldwide, especially in developing countries and low-income areas where half of the affected population live (Bousquet et al, 2007). However, allergic conditions are still more prevalent in the industrialized world than in developing countries and in urban centers than in rural towns (Cooper et al, 2008; The ISAAC steering committee, 1998). It is estimated that asthma and allergy affect together 700 million people around the globe (Bousquet et al, 2007; Bateman ED and Jithoo A, 2007) with an estimated global prevalence of 11.3% (The ISAAC steering committee, 1998). Just in Canada, respiratory diseases provoke important economic impact representing 6.5% of total health-care costs; i.e, 5.7 and 6.72 billion Canadian dollars in direct and indirect costs, respectively (GARD, 2008).

Since immune responses to both allergens and helminth infections induce type-2 cytokine responses [12, 20] and the subsequent mechanisms, it may be argued that allergic inflammation may be a mechanism developed by the host to control helminth infections, although during chronic infections these responses may be down-modulated [20] to protect the parasite from elimination and the host from damaging pathology [12].

Numerous epidemiological studies have provided evidence for an inverse association between geohelminth infections and the occurrence of allergy [12, 34, 35, 36, 37, 38]. An understanding of the immunologic mechanisms by which chronic helminth infections suppress allergic inflammatory response not only to helminth allergens but also inhalant allergens, may lead to the development of novel therapeutic interventions against allergic and other inflammatory diseases [20]. One of these mechanisms might be the induction of mT_H2 responses

which have been described only for cat exposures in human populations and during experimental murine helminth infections [26, 31]. However, to the best of our knowledge no evidence for such a phenomenon in human helminth infections has been made public. In the present study we have investigated the presence of a modified T_H2 response in humans suffering chronic infections with *Ascaris*; a fact that could explain the divergent association between geohelminth infections and allergic disorders.

Hypothesis

Parasite-specific cytokine profiles of individuals with chronic *Ascaris lumbricoides* infections are consistent with modified Th2 responses, which are characterized by high type 2 cytokines and IL-10 levels with concomitant increase in the levels of circulating parasite-specific IgG 4 antibodies. This response is different from the one observed in individuals with acute infection which is characterized by high levels of T_H2 cytokines but low levels of IL-10 and low levels of IgG 4.

Objective

1. To define baseline socio economic and biological characteristics of the studied population.
2. To characterize *Ascaris*-specific and nonspecific immune response in school-aged children suffering from chronic ascariasis and compare them to the responses evoked in acutely infected and non infected children.

- 2.1. Determine humoral immune responses including polyclonal IgE and parasite-specific total IgG (i.e., all subtypes), IgG4 and IgE in children having chronic, acute or no infections with roundworms.
- 2.2. Determine cytokine production in stimulated peripheral blood mononuclear cells isolated from children with chronic, acute or no infection with *A. lumbricoides*.
3. To explore whole blood transcriptomic features in all studied children.

Materials and Methods

Study population and design

We conducted a cross sectional study in rural areas of the tropics of Ecuador from May to October, 2008 to determine the immune response in children infected with *Ascaris lumbricoides*. Schoolchildren aged 7 to 12 from two helminth endemic rural areas located in northwestern Ecuador [4, 39, 40] were selected. Geographical coordinates were N0°20'/N0°30' and W79°15'/79°30' for Area 1 whereas for area 2 were N 1°0'/N1°30' and W78°45'/W79°0'. Communities in Area 1 were close to Las Golondrinas town in the District of Quinindé while Area 2 was composed of three communities, namely La Alegría, Santa Lucía y El Pampanal de Bolívar, in the Districts of Eloy Alfaro and San Lorenzo, all Districts were located in the Province of Esmeraldas. A validated questionnaire [40] was applied to guardians and was used to collect data on important socio-economic characteristics of the studied population and subjects were asked to provide three consecutive stool samples once and a final stool sample three months apart. At the moment of stool sample collection, blood

samples were obtained for PBMCs isolation and further *in vitro* cell culture and genome-wide microarray analyses (see below). Inclusion criteria were: written informed parental consent, ages 7 to 12 years (figure 2). To establish non-infected status, all stool samples from individuals had to be negative by three different techniques (Fresh, Kato-Katz and ethyl-acetate concentration) (Table 1).

5.2 Stool analyses

Parasite presence in stool samples was assessed using three techniques: direct saline examination, modified Kato-Katz and formol-ethyl acetate-concentration methods [49]. Direct examination was carried out by mixing one drop of isotonic saline solution with faecal sample on a glass slide and examined by microscopy. For the Kato-Katz test, faecal samples were passed through a wire mesh and were placed onto a plastic template to measure the amount of material. After removal of the template, the sample was overlaid with a glycerol embedded cellophane membrane, observed under the microscope and ova concentration was determined as previously described [16]. Formol-ethyl acetate concentration technique was carried out as previously described [49]. Faecal samples were forced through gauze, 1 ml of the filtrate was transferred to a conical tube which received 9 ml of 10% formaldehyde plus 3 ml of ethyl-acetate, mixture was shaken vigorously and tubes were centrifuged at 2000 rpm for 3 minutes before sediment was suspended in 1 ml of saline and analysed by light microscopy. Two slides were examined for each faecal sample by two different technicians.

5.3 Serum antibodies measurement

Seven ml venous blood samples were collected from forearm in tubes with sodium heparine as anticoagulant. Blood samples were centrifuged and plasma was frozen until further use. Plasma stored at -20°C was used to measure antibodies against *A. lumbricoides* (Cooper et al, 2000; Cooper et al, 2003). Briefly, a 96 well microtiter plate (Corning, high binding) was incubated overnight with a PBS-soluble extract of adult female *A. lumbricoides* worms obtained as previously described [16]. After blocking with 150 µl of blocking buffer (PBS 1X, Tween 20 [0.05%], BSA 0.25%) plates were washed 6 times using PBS-Tween 20 (0.025%) and 100 µl of standards and diluted samples (1:200 and 1:10 for total IgG and IgG 4, respectively) were added to the plate, washed as before and followed by addition of 100 µl of the diluted (1:500) anti-immunoglobulin antibody (Goat α-human IgG Fc-AP for specific total IgG, and mouse α-human IgG4 for specific IgG4, Jackson ImmunoResearch, PA, USA). Plates were washed as before and were then developed by adding phosphatase substrate (Sigma) for IgG and in the case of specific IgG4 an extra step using 100 µl of diluted (1:500) goat-α mouse IgG-AP (Jackson ImmunoResearch, PA, USA) was required before washing as indicated above with the subsequent addition of substrate (FEIA). Values were expressed in arbitrary units based on OD since for these immunoglobulins there is no universally accepted standardized ELISA assay.

Standards for these assays were obtained from 15 uninfected individuals who had previously been shown not to harbor *Ascaris* infection and mean OD values were obtained [16]. Samples were considered positive if OD values were above mean + 3 SD of standards.

Ascaris-specific and polyclonal IgE antibodies were measured by fluorometry using the UNICAP assay at the Centre for Allergic Diseases, University of Virginia, Charlottesville, Virginia, following the manufacturer's instructions (Pharmacia, Sweden) as previously described [4]. Polyclonal IgE was originally measured in IU/ml, anti-*Ascaris* IgE in KU/l and the limit of detection for these assays were set at 35 IU/ml (35 KU/l in the case of anti-*Ascaris* IgE).

5.4 PBMCs isolation

Seven ml of venous blood samples were drawn into Vacutainer tubes containing sodium heparin and PBMC's were isolated following the described procedures. Briefly, 7 ml of blood were centrifuged at 1750 rpm at 20°C for 30 minutes in a 15 ml conical tube containing 5 ml Histopaque Lymphocyte Separation Medium (Sigma). Mononuclear cells were transferred to a new 15 ml conical tube to which RPMI medium (Biowhitaker, MD, USA) was added to a final volume of 15 mls. Tubes were centrifuged at 2000 RPM for 10 minutes at RT and the pellet was resuspended in 1 ml of RPMI containing 5% Bovine Foetal Serum (Biowittaker, MD, USA), 1% HEPES (Biowittaker, MD, USA), 0.08 mg/ml of gentamicin (Life Technologies, Gibco, BRL) and 2 mM/L L-glutamine (Biofluids). Cells were counted in a haemocytometer and viability was assessed by addition of trypan blue.

5.5 PBMC culture and in vitro stimulation

Two hundred and fifty μ l of cell suspension, at a concentration of 4×10^6 cells/mL were transferred to wells in a Cellstar[®] tissue culture plate (Greiner-Labortechnik). An additional 250 μ l of supplemented RPMI medium (RPMIc)

containing *A. lumbricoides* antigen (10 µg/mL [16]) or medium alone were added. Plates were cultured at 37°C in an environment containing 5% CO₂ for 5 days. Supernatant fluids were collected and stored at -70 °C. The samples were shipped to the UK on dry ice and stored at -70 °C before cytokine production measurement at St George's University of London.

5.6 Cytokine and chemokine production measurement

Cytokine levels (IL-5, IL-10, IL-13 and IFN gamma) from day 5 culture supernatants were measured using fluorometric xMAP Luminex technology and commercial kits (R&D LUB000, LUH000, LUB213, LUH217 and LUH205) as described previously [46]. This technique uses fluorometric color-coded beads which have cytokine-specific immunoglobulin on their surfaces (different colors for different cytokines), samples are transferred to wells where they interact with thousands of different beads, specific binding is achieved and read with the use of a two laser (laser one reads the identity of the bead and laser two reads intensity of binding) instrument. An in-house 19-plex assay from the Centre for Infection at St. George's University of London was used to measure other cytokines and chemokines (IL-1 α , IL-1 β , IP-10, IL-16, GM-CSF, RANTES, IL-4, IL-2, IL-12, IL-15, TGF- β , SDF-1 β , MCP-2, MIP-1 α , MIP-1 β , TNF- α , IFN- β , IFN- γ and MIG). Procedure was performed following manufacturer's instructions. Briefly, Filter-bottomed microplates were pre-wetted with wash buffer, vacuum-filtered loaded with specific beads and 50 µl of samples and standards were added. Plates were sealed with aluminium foil and incubated using a microplate shaker at 500 rpm, at room temperature for 3 hours (2 hours in the case of IL-13). Plates were vacuum-filtered and washed 3 times before adding 50 µl of

diluted biotin antibody cocktail to each well and then were subsequently incubated for another hour under same conditions. Plates were vacuum-filtered again and washed as before 3 times before adding 50 µl of diluted streptavidin-PE to each well, and incubated for an extra half an hour as before. Finally, plates were vacuum-filtered, washed 3 times and samples and standards were resuspended in 100 µl of washing buffer before reading. Plates were read using a BIO RAD Luminex reader. Standard concentrations vary according to the batch used, thus every assay requires new standard concentrations detailed in the Standard Value Card which comes with each kit. Standard curves of known concentrations (Standard Value Card) of human cytokines were used to convert median fluorescence intensity (MFI) into cytokine concentration in pg/ml.

5.7 RNA isolation

Blood samples (2.5 ml) were drawn into PAXgene tubes (Preanalytix, GmbH), left at room temperature for two hours and stored frozen at – 70 °C. Tubes were thawed and afterwards centrifuged at 10 °C, 4000 rpm for 10 minutes, supernatants were discarded, 4 ml of RNase free water was added, tubes were vortexed for 1 minute and centrifuged again at 10 °C, 4000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 ml of RNase free PBS, suspension was transferred to 1.5ml tube, and centrifuged to 4000 rpm for 10 minutes. Supernatant was eliminated and the pellet was homogenized with 700 µl of Qiazol lysis reagent (Qiagen) and a 1 ml disposable syringe, mixture was incubated at RT for 25 minutes before subjecting it to chloroform extraction, aqueous phase was mixed with 1.5 volumes of 100% ethanol (RNase free) and transferred to a spin column (Qiagen microRNA easy

kit), centrifuged at 10000 rpm for 15 seconds at RT, 350µl of RWT buffer were added to the column and centrifuged at 10000 rpm, for 15 s at RT and, DNase was added to the column then the column was washed with 500µl of RPE buffer by centrifuging at full speed (approximately 12000 rpm) for 15 s and then for 1 minute at, room temperature. Finally, the spin column received 50 µl of RNase free water, the column was placed on top of a new 1.5 ml centrifuge tube, and RNA was eluted by centrifugation at 12800 rpm at room temperature, this step was repeated and eluates were frozen at – 70 °C. RNA Quality Control (Integrity and concentration) was assessed using a bioanalyzer® and a Nanodrop® equipment, respectively.

5.8 Microarrays

Gene expression levels were measured by microarray analyses using Illumina Human 6 version 2 chip (Illumina, inc), which includes more than 48000 probes with a genome-wide coverage.

These experiments were conducted with a first step of RNA amplification using the Illumina® TotalPrep™96 Kit (Patent Pending). First, samples were reverse-transcribed with an oligo(dT) primer bearing a T7 promoter and using ArrayScript™ reverse transcriptase. A second strand synthesis was made from cDNA, this new strand formed a template for in vitro transcription with T7 RNA polymerase. Biotinylated, antisense RNA copies of each mRNA (or microRNA) were hybridized with the corresponding Illumina arrays and read using Bead Station 3.1.

Microarray data was analysed using Bioconductors and Genespring software. Clustering of genes into different pathways and according to ontology was made using web-based softwares: GeneFinder, Bioinformatic Harvester and Innate Data Base (University of British Columbia, Vancouver, Canada). Finally, we used Innate Data Base to look for interactions of genes involved in innate immunity and to define innate immune pathways that were over represented in the samples.

5.8 Statistical analyses

Descriptive statistics of all variables were performed using Graph Pad Prism 4.0 or SPSS 15.0 softwares on a Toshiba Equium A200-1V0 computer. Non-parametric tests were used to compare cytokine and antibody levels between groups classified as either binary variables (chi-squared test) or continuous variables (Mann-Whitney test for two-group and Kruskal-wallis for three group comparisons). Post hoc tests (Dunns test) were used to correct p values for multiple comparisons. Principal components analysis was used to reduce multiple cytokine parameters to 3 principal components (to reflect latent variables such as T_{H1} , T_{H2} , and mT_{H2}).

Antibody data was analysed using Kruskal-Wallis test with Dunn's multiple comparisons as post hoc test. We calculated two different antibody ratios: specific IgG4 to specific IgE ratio and specific IgE to polyclonal IgE ratio. These ratios will allow us estimate the importance of the modified Th2 response which should have greater IgG4 to IgE ratios reflecting IgG4 induction with lack of IgE sensitisation. However, since immune response to *Ascaris lumbricoides* is characterised by strong IgE production it is expected to find increased parasite-

specific IgE which would be reflected by the specific to polyclonal IgE ratio. To analyse cytokine data, we subtracted spontaneous cytokine production from stimulated values. Negative results were assigned a value of zero. Values lower than the assay's limit of detection (LOD) were assigned half of the corresponding LOD for the concerned assay. In order to discern statistical differences we conducted the following statistical analysis: descriptive statistics, Kruskal-Wallis tests for comparisons of absolute values, Chi square test for comparisons of the proportion of responding individuals.

To further investigate cytokine expression across all groups, we run a Factor Analysis using a Principal Components Analysis extraction method. To normalize values we included \log_{10} transformed data of cytokine expression levels [T_H1 cytokines (IL-2, IFN- γ and TNF- α), the T_H2 cytokine (IL-5) and the immunomodulatory cytokine (IL-10)] after *Ascaris* antigen stimulation. We excluded IL-13 production levels from these analyses given the reduced sample size for this measurement (n = 3 for Group 1; 11 for Group 2; and, 6 for Group 3). All components were set up to have eigen values over 0.9 and subjected to Kaiser-Meyer-Olkin measures, Barlett's test of sphericity and Sampling Adequacy tests.

Strictly exploratory gene expression analyses were done as follows. Genespring software was used to perform quality control of data and genes were considered to be differentially expressed if a 1.5 fold difference in expression was observed with p values lower than 0.05. The Bioinformatic Harvester and Innate Data Base (www.innatedb.ca) on-line servers were used for data mining to explore gene function. Finally, data was uploaded into Innate Data Base to investigate important gene interactions and to identify genes with altered gene

expression. Pathway and ontology over representation analyses were performed to identify pathways (or ontologies) which are significantly associated with differentially expressed genes [50]. The restrictions imposed to do this analysis were: 1) A threshold for gene expression fold change of 1.5; 2) a threshold for associated p values of 0.05; 3) the use of hypergeometric statistical method of analyses; and, 4) the use of Benjamini and Hochberg correction for multiple testing.

Finally, we uploaded into innate database a tab delimited file containing all comparisons (i.e.; Group 3 vs. 1; Group 3 vs. 2; and, Group 2 vs. Group 1) in order to have a profile-like view of genes behaviour and to analyse the pattern of gene expression within pathways across comparisons.

Results

Baseline characteristics of study population

Children lived in two different zones, both in Esmeraldas Province in the north of Ecuador (Figure 3). Zone 1 was comprised of several satellite communities to the rural town Las Golondrinas. Zone 2 was located in the north boundaries of the Esmeraldas province where three rural communities were selected for screening on the basis of previous data.

A total of 1038 children (95% of 1091 children) were screened for helminth infections and/or anaemia (Fig. 2). Six hundred and twenty three children were eligible based on inclusion criteria. Children were allocated in three different

groups according to the criteria indicated in Table 1 and Figure 2. Out of three hundred and thirty five children who were negative for helminth infections, 41 showed no serological evidence of *A. lumbricoides* exposure and were considered as group 1 (uninfected) individuals. Meanwhile, 288 children were infected with *A. Lumbricoides*. Infected children were further classified according to the absence (45 Children) or presence (61 Children) of serum *Ascaris*-specific IgG4 and were assigned for group 2 (“acute” infections) and 3 (“chronic” infections) respectively.

Children’s socioeconomic and biological baseline characteristics are summarized in table 2.

There were differences in ethnic composition between the groups with African descendants being especially frequent in group 3 whereas sex and age were not statistically different. Improper biological waste disposal was more frequent in Group 3 children. Crowding was statistically associated to *Ascaris* infection status. Higher level of education of the mother, father, and children seemed to be associated to lower prevalence of *Ascaris* (table 2). Major nutritional status was inferred from Body Mass Index calculation and anaemia prevalence within groups (table 2). We found no statistical difference between groups but there was a trend of having more anaemia in Group 3 and lower nutritional status as *Ascaris* infection status increased (i.e., from Group 1 to Group 2 and to Group 3). Intensity of *Ascaris* infection measured by FEC was higher in Group 3 children than in Group 2. Additionally, All Group 3 children and 65% of children in Group 2 were co-infected with whipworms ($p < 0.05$, chi square test). Table 2 also shows time elapsed since last anthelmintic treatment on which we observed no differences among the groups.

Immune responses according to intensity of infection.

6.2.1 Humoral Immune response

An important host defence to parasitic infections is the synthesis of IgE. Accordingly serum concentrations of this immunoglobulin were measured. Total IgE was statistically higher in Group 3 than in groups 2 and 1 (Figure 4A). Similar results were observed for the concentrations of specific anti-*A. lumbricoides* IgE (Figure 4A).

Since the presence of IgG4 was used to allocate participating children in group 3 and the absence of anti-*A. lumbricoides* total IgG (i.e., subtypes 1-4) was used to select group 1 children, there were differences in these antibody concentrations that reflect this selection. Figure 4B shows the concentration of IgG subtypes among the different groups. Anti-*A. lumbricoides* IgG4 was higher in group 3 than in groups 2 and 1. Similarly, serum levels of total anti-*A. lumbricoides* IgG were higher in group 3, although the differences were not statistically significant.

Considering that one of the characteristics of a mTh2 response is an increase in the levels of specific IgG4 relative to specific IgE, the ratio of IgG4:IgE was estimated. Table 3 summarizes the findings for antibody ratios calculations. There were significant differences in the specific IgG4 to specific IgE ratio across groups, with the ratio being greater in group 3 compare to groups 1 and 2. There were no inter-group differences for the specific to polyclonal IgE ratio.

6.2.2 Cytokine expression profiles

Cytokine production was determined from supernatants after culturing PBMCs with or without antigen stimulation. Different cytokines representing T helper polarization were measured (i.e., Th1, Th2 and immunomodulatory cytokine, IL-10).

The Th1 cytokines included for determination were IL-2, IFN- γ , TNF- α . Initially, spontaneous cytokine production was evaluated. Table 4 shows the median and the percentages of responding children of the different evaluated cytokines. The median concentration of IL-2 was increasingly higher from Group 1 to Group 3 and the differences were statistically significant. The number of children with detectable IL-2 production was lower in Group 1 than in groups 2 or 3. Group 3 children presented the higher median IFN- γ production albeit statistical association was weak. However, the numbers of responding children in each group was similar (Table 4). Finally, there were non statistical differences in TNF- α spontaneous production by PBMCs among the groups (Table 4). We then analysed T_H1 cytokine production in stimulated PBMCs (Figure 5). Median IL-2 production after SEB stimulation in Group 1 was higher than groups 2 and 3. The number of responders to the antigen challenge was 100% both in Group 3 and 1 while only 80% of children responded with detectable IL-2 production to SEB stimulation. When cells were stimulated with PPD, the median concentration of IL-2 was greater for Group 1 children than the median for group 2 ($p < 0.05$) and 3 ($p > 0.05$). Similarly, the number of responders to PPD was higher in Group 1. A similar pattern of median IL-2 production in response to PPD stimulation was observed when PBMCs were challenged with *A. Lumbricoides* antigens, albeit this time differences were not statistically significant. There were non important differences in either median

concentration or percentage of responding individuals when IFN- γ production was analysed in either SEB or PPD-stimulated cells. However, *Ascaris* antigen stimulation induced more children in Group 1 to respond with detectable levels than in the other groups albeit differences were not statistically important. There were no differences in the percentage of responding individuals when TNF- α production was analysed in SEB or PPD stimulated PBMCs. However, although non statistically significant, median TNF- α production was higher in both Group 2 and Group 3 as to compared to Group 1 children after PPD stimulation. If stimulation with *Ascaris* antigens was considered, all differences were non-statistically significant. However, when the proportion of responding individuals was analysed it was found that less Group 2 children responded with TNF- α production when compared to both Group 1 and Group 3 individuals ($p=0.08$, chi square test).

Spontaneous production of Th2 cytokines (IL-5 and IL-13) was as follows. IL-5 production was more than 20 times higher in Group 3 children when compared to both Group 1 and Group 2 children and the difference was statistically significant (Table 4). Similarly, the number of children responding with detectable IL-5 production was higher in Group 3 compared with the other groups. IL-13 median production was below the limit of detection for all groups although only in Group 3 there were cells from children that spontaneously produced IL-13. Stimulated production was as follows (Figure 6). Median IL-5 production was higher in Group 3 both in PPD and *Ascaris* antigen-stimulated cultures, although differences were not statistically significant. Similarly, median IL-13 production was greater in Group 3 after PPD and *Ascaris* antigen stimulation (non statistically significant differences). Moreover, the percentage

of children responding with detectable IL-13 was higher in Group 3 than in the other groups.

Due to the modulatory characteristics of IL-10 [19] the expression of this cytokine is considered apart. Thus, spontaneous IL-10 production was greater in Group 3 children ($p < 0.05$) while the percentage of children responding with detectable IL-10 after both PPD and *Ascaris* antigen stimulation was higher in Group 3 than in the other groups (Figure 7). Moreover, median IL-10 production in *Ascaris*-stimulated cultures was higher in Group 3 children than in Group 2 ($p > 0.05$) and Group 1 ($p < 0.05$) (Figure 7).

6.2.4 Induction of a modified Th2 response as defined by a Principal Component Analysis.

Using the data reduction techniques known as Factor Analysis and Principal Component Analysis, we explored patterns of cytokine expression among individuals and then tested if these patterns were segregated in the different groups. The rotated analysis yielded 3 components, with Eigen values over 0.9, explaining 91% of variance between data, as shown in Table 6. The components and their loading values are shown in table 5; the first component was called T_H1 component since it was strongly associated with the expression of IFN- γ and TNF- α ; the second component was called modified T_H2 (mT_H2) since it was related to the expression of both IL-5 and IL-10 and the third component was referred to as T_H2 as IL-2 and IL-5 levels were positively associated while IL-10 was negatively associated to this component. One limitation of this analysis was that when performing the Kaiser-Meyer-Olkin test for sampling adequacy we obtained a low value of 0,492; though Barlett's Test

of Sphericity let us conclude that the variables were correlated ($p < 0.001$), as shown in appendix I. We then analyzed the fitness each variable had to the structure defined by the other variables. As shown in appendix I, IL-5 and IL-10 did not adjust to the model since they had a Measure of Sampling Adequacy value lower than 0.05 [51]. However, when analysing communalities we found that the percentage of common variance in the extracted factors is superior to 50% for every single variable analysed (appendix I). We afterwards calculated values for each component in the individuals and compared components across groups. In figure 8 we can observe the comparison of mT_H2 component scores among the different groups.

6.2.3 Chronic Helminth infections are associated with an increased risk of a modified T_H2-like response defined by Factor Analysis using a Principal Component Analysis extraction

We then analysed if chronic helminth infections were associated with an increased risk of responding with a modified T_H2 response as defined by the component scores obtained after a Factor Analysis using a Principal Component Analysis Extraction Method. To do this we classified individuals as having (mT_H2 Component Scores higher than 0) or not having (mT_H2 Component Scores 0 or lower) a modified T_H2 response and afterwards we calculated odds ratio.

As shown in Figure 9, we found that being chronically infected with *Ascaris lumbricoides* was associated with an increased risk of having a modified T_H2 response when compared to uninfected controls (OR = 5.2, 95% CI = 1.25-21.58; $p = 0.02$) or to acutely infected individuals (OR = 2.4, 95% CI = 0.65-9.13; $p = 0.18$). Moreover, albeit the difference was not statistically

significant, children acutely infected with roundworm had a 2 times higher risk (OR = 2.1, 95% CI = 0.55-8.26; p = 0.27) of responding with a modified T_H2 response when compared to uninfected endemic controls.

6.2.4 Ethnic backgrounds were not associated with cytokine production.

Since the ethnic composition was different in Group 3, we tested whether ethnicity was associated with the outcomes measured; i.e., cytokine's expression levels for IL-2, IL-5, IL-10, IL-13, IFN- γ and TNF- α after culturing PBMC's with *Ascaris* antigen. Data shown in table 7 demonstrates that ethnicity was not associated to cytokine expression when culturing PBMCs with parasite-specific antigens.

6.3 Genome-wide gene expression profiles.

6.3.1 Overall differential expression

Out of more than 48000 probes analysed, we found a total of 52 genes whose expression was differentially regulated in the three different groups of individuals (Figure 10).

6.3.2 Group 3 plus Group 2 to Group 1 comparison (Worm Infection Effect)

One of the different expression profiles found included genes that were up or down regulated in both Group 3 and Group 2 children when compared to Group 1 children but with no difference in the expression if Group 2 and Group 3 were compared. We term this profile "worm infection effect" since it included genes up (or down) regulated during both "acute" and "chronic" infections. Figure 11 shows this profile and

table 8 shows the most relevant genes having this profile of expression, a complete table might be found in appendix II.

6.3.3 Group 3 to Group 1 plus 2 comparison (Chronic effect)

Genes differentially expressed (up or down-regulated) when comparing chronic infections vs. acute or no infections show a pattern of expression we named Chronic Effect. In this pattern there is no difference in the expression between Group 2 and Group 1 children. A schematic representation is shown in figure 12 and table 9 contains the most relevant genes, a more comprehensive list may be found in appendix II.

6.3.4 Chronically infected vs. uninfected controls.

In this case, genes are either up or down regulated during chronic/high burden infections when compared to uninfected controls, and this difference is statistically significant. However, the difference in expression when comparing Group 2 to any of the other two groups is not statistically significant.

Table 10 shows all up-regulated genes for this comparison while in Table 11 we find down-regulated genes, only the most immunologically important results are shown. The rest of genes are shown in appendix II.

6.3.5 Biological context analyses

6.3.5.1 Ontology Over Representation Analysis

To gain more in-depth interpretation of gene expression analysis in a network and pathway context, we ran an Ontology Over Representation

Analysis using Innate Data Base (University of British Columbia, www.innatedb.ca). This kind of analysis permits the extraction of the main ontologies that are represented in the uploaded list of genes. It therefore permits the inference of which processes are regulated in response to *Ascaris* infection status [50].

Table 12 to 14 shows the results of these analyses. Group 3 to Group 1 comparisons results are shown in Table 12. Table 13 shows differentially regulated ontologies observed when comparing Group 3 vs Group 2. Immunologically interesting ontologies are shown, a complete table might be found in appendix III.

Table 14 shows differentially regulated ontologies observed when comparing Group 2 vs. Group 1. A full table showing all ontologies is provided in appendix III.

6.3.5.2 Pathway Over Representation Analysis

Pathway Over Representation Analysis (innatedb.ca) allowed us to define which pathways are associated to differentially expressed genes; i.e. how many genes within a pathway are differentially expressed in the different groups. The latter would provide an overall picture of the immunological and metabolic pathways that are affected by *Ascaris* infection. As before, here we show the most interesting results, complete data sets for each comparison may be found at appendix IV.

Group3 to Group 1 comparisons showed that several immunological pathways were affected by *Ascaris* infection. Interestingly, among up-

regulated pathways during chronic infection we found T Cell Receptor and IL2 signalling pathways (for a more comprehensive list see Table 15). Table 16 shows down regulated pathways when comparing Group 3 to Group 1.

We found TCR, BCR and TGF-B receptor signalling pathways' genes being up regulated during chronic ascariasis when compared to acutely infected children (Figs 13, 14 and 15 showing these genes and genes with which they interact). As explained before, just one gene, SOS1, is responsible for almost all the up regulation in pathways observed when comparing chronic vs. acute infection (corrected p value > 0.05, except for the first pathway) (table 17). No pathway was down regulated when these groups were compared.

Table 18 shows up-regulated pathways when comparing Group 2 vs. Group 1. No pathway was down regulated in this comparison.

Discussion

Immunity to Ascariasis

In the present report it was found that children infected with *Ascaris lumbricoides* showed slightly higher TNF- α production than uninfected controls ($p > 0.05$). Previously, it has been shown that worm infection is positively associated with TNF- α responsiveness to TLR4 stimulation [24]. Increased TNF- α production could be indicative of a non-inflammatory context being set up during ascariasis since, although still controversial, TNF has been shown to

participate, when co-expressed with iNOS by DCs, in IgA class switching, which is an immunoglobulin known for performing its functions in a non-inflammatory context [52]. *Ascaris* infected children showed increased production of T_H2 cytokines which despite of not being statistically significant was consistent with previous reports showing T_H2 polarization during *Ascaris* infection [16]

Reports of IL-10 production during ascariasis have shown inconsistencies. Baseline IL-10 production based on the percentage of responders was lower in infected children ($p < 0.0001$) whereas parasite-specific IL-10 production was weakly and positively associated to *Ascaris* infection. Some studies have shown a positive relationship between ascariasis and IL-10 production [18, 53, 54] and others have reported a negative one [55]. One explanation for these contradictory results may be different burdens and length of exposure, as well as different concomitant whipworm co-infection rates.

7.2 Induction of a modified T_H2 response during chronic ascariasis

The modified T_H2 response is an immunological phenomenon deviating from originally IL-4-induced classic T_H2 polarization to a new down regulating phenotype, probably due to IL-10 activities (Figure 16). This response is characterized by high IL-10 production, high levels of circulating specific-IgG4 and IgG antibodies with relative low amounts of specific-IgE levels [28]. Table 19 show the differences found in the different T_H2 responses described so far.

We found that children chronically infected with *Ascaris lumbricoides* responded with an immune phenotype resembling the modified T_H2 response showing high specific IgG4 levels and the typical T_H2 cytokine profile. Moreover, increased IL-

IL-10 production suggests the induction of a modified T_H2-like response (Table 19). This was demonstrated by the significant differences observed when comparing PCA-extracted mT_H2 component between group 3 and group 1 (OR = 5.2, 95% CI = 1.25-21.58). These data suggest that in children living in the rural Tropics of Ecuador helminth infections, specifically *Ascaris lumbricoides* infections induce modified T_H2 responses. To the best of our knowledge this is the first time that it is demonstrated a helminth-induced T_H2 modified response in humans; nonetheless, a modified T_H2 response in murine models has been described [20, 31].

Modified Th2 responses may have putative protective roles which would rely on IL-10 production and on IgG4 class switch induction. It has been reported that IgG4 may block IgE-mediated harmful effects [23, 56, 57]. It has been suggested that IgG4 antibody isotype would play a role in roundworm susceptibility [57]; however, IgG4 could just be a by-product of chronic helminth infection-induced immune modulation, since it has been shown that IL-10 induces a switching to this isotype [57] and IL-10 has been associated with chronic helminth infections [20]. This modified T_H2 response although induced by helminth antigens may affect the response to other antigens including allergens [31]. Since the modified T_H2 response was observed only during chronic infections this data may help us understand better why acute infections with helminths, especially during larvae migration, are associated with increased allergic manifestations while chronic infections are inversely associated with allergy. Accordingly, anti-inflammatory cytokines such as IL-10 and TGF- β would be produced after immune system continual stimulation with parasite antigens, just like the observed with cat allergen stimulation.

7.3 Gene expression signatures: an exploratory approach to the genetic component in the modified T_H2 responses

Ascaris infection appeared to up-regulate of CCL23 an IL-4 and IL-13 induced CC chemokine that has activity on resting T lymphocytes, monocytes, neutrophils, osteoclast precursor cells and endothelial cells [47]. Chronic ascariasis was positively associated with the expression of SOS1 gene which encodes for a Guanidine Nucleotide Exchange Factor (GEF) for RAS proteins [58] this finding is consistent with up-regulation of T Cell Receptor pathways during chronic infections (corrected p values > 0.05).

Up regulated genes during chronic infections included HLA-DRB1. This might propose a genetic component in the differences observed and which is consistent with previous findings showing that individuals carrying the allele 0701, a DRB1 allele, produced higher amounts of IL-10 in response to a Fel d 1 epitope in the context of a modified Th2 response [30]. Other up regulated genes were IFN- γ -induced antimicrobial protein (INDO), the mucosal protective protein known as Trefoil Factor 3 (which could be suggestive of mucosal healing networks being activated during roundworm infections).

Chronic *Ascaris* infection induced down regulation of genes coding for important regulators of inflammatory molecules and metabolic pathways such as ANXA3 (inhibitor of phospholipase A2), CYP4F3 (involved in Leukotriene B4 degradation), IL-8 (a proinflammatory chemokine), Fc γ RIIIA, IL-1RII (an IL-4 induced decoy receptor), Toll like-receptor 6 (TLR-6) and Vanin 2 (a protein participating in transendothelial neutrophil migration), HLA-DQ and CREB5. Ontologies and pathways associated to these genes were numerous but due to

lack of statistical power p values were greater than 0.05. It is very important to emphasize that correction for multiple comparisons would be conservative given the high number of probes and the small number of individuals analysed. Nevertheless, T-Cell Receptor signalling pathway was significantly up-regulated during chronic infections when compared to acutely infected children (corrected $p < 0.05$).

Taken together these data suggest that although *Ascaris* infection is followed by activation of both immune system (CCL23; T cell receptor signalling pathway, ANXA3 and CYP4F3 down regulation) and healing mechanisms (i.e. mucosal protective trefoil proteins), chronic ascariasis may induce regulation in the gene expression of immune mediators (IL-8, FcγRIIIA, IL-1R2, Vanin2, TLR-6). This gene expression pattern would be concomitant to the induction of a modified T_H2 response. However, the ability of an individual to respond with high IL-10 production to specific stimuli could depend on specific HLA haplotypes, an issue that correlated with the increased expression of HLA-DRB1 in group 3 children but that we did not further investigate. Moreover, it seems that during chronic infection an overall non-inflammatory context is set up as suggested by higher TNF- α production (which in turns induces IL-10 production and IgA class switching in mucosal surfaces [52]).

7.4 Study limitations

One important limitation of this study is the small sample size ($n=20$, per group), from which we think that important differences might have been overlooked because of lack of statistical power. Nevertheless, the study population was similar with respect to potential confounding variables such as sex, monthly

incomes, recent ant-helminthic treatment, anaemia and nutritional status defined by Body Mass Index. On the other hand, some other variables like concomitant whipworm infections, waste disposal procedures and parent literacy were not taken in account and could confound some of the associations observed.

Since this was a cross-sectional study, no strict categorization of individuals as being acutely or chronically infected may be done. This is why we used terms “acute” or “chronic” with quotes in this manuscript. However, it is known that persistent allergen stimulation during successful immunotherapy leads to IgG4 class switching [48] and that clinical silent, helminth-infected individuals who also usually develop chronic infections, have increased IgG4 responses [48]. Moreover, chronic helminth infections as well as successful immunotherapy are associated with high levels of IL-10, which is known to induce B cells to produce IgG4 rather than IgE antibodies [12, 48]. Thus, although the study design do not allow us to ascertain chronicity of infection we have indirect evidences suggesting that some children may have been suffering from chronic *Ascaris* infections.

Finally, the impact of possible contact with Th1-promoting stimuli like BCG vaccination (routinely applied to all newborns in Ecuador) or environmental/pathologic mycobacteria exposure was not registered and thus not analysed.

Conclusions

To the best of our knowledge this is the first time that it is demonstrated a helminth-induced T_H2 modified response in humans although we lack statistical

power in the present report. However, we consider that these results are encouraging and future work is needed in order to clarify whether the modified T_H2 response is a common feature of chronic ascariasis.

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